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Report

Topographically Distinct Epidermal Nociceptive Circuits Revealed by Axonal Tracers Targeted to *Mrgprd*

Mark J. Zylka,[2,3] Frank L. Rice,[1] and David J. Anderson[2,3,*]
[1]Center for Neuropharmacology and Neuroscience
Albany Medical College
Albany, New York 12208
[2]Division of Biology, 216-76
[3]Howard Hughes Medical Institute
California Institute of Technology
Pasadena, California 91125

Summary

The brain receives sensory input from diverse peripheral tissues, including the skin, the body's largest sensory organ. Using genetically encoded axonal tracers expressed from the *Mrgprd* locus, we identify a subpopulation of nonpeptidergic, nociceptive neurons that project exclusively to the skin, and to no other peripheral tissue examined. Surprisingly, *Mrgprd*$^+$ innervation is restricted to the epidermis and absent from specialized sensory structures. Furthermore, *Mrgprd*$^+$ fibers terminate in a specific layer of the epidermis, the stratum granulosum. This termination zone is distinct from that innervated by most CGRP$^+$ neurons, revealing that peptidergic and nonpeptidergic epidermal innervation is spatially segregated. The central projections deriving from these distinct epidermal innervation zones terminate in adjacent laminae in the dorsal spinal cord. Thus, afferent input from different layers of the epidermis is conveyed by topographically segregated sensory circuits, suggesting that at least some aspects of sensory information processing may be organized along labeled lines.

Introduction

The interface between the brain's perception of the external world—exteroception—and that of its internal world—interoception—is the skin, the body's largest sensory organ. Sensory information is initially transmitted from the skin to the brain by the fibers of primary sensory neurons located in trigeminal and dorsal root ganglia (DRG). These neurons comprise a heterogeneous population including mechanoreceptors and nociceptors. Nociceptive neurons detect noxious thermal, mechanical, and chemical stimuli that can evoke the sensation of pain. The peripheral endings of these neurons innervate a variety of cutaneous targets, including hair follicles, Merkel cells, Meissner's corpuscles, blood vessels, and epidermis. The central projections of these neurons terminate in a relatively restricted region of the dorsal horn in the spinal cord. This region includes lamina I, the most superficial layer, as well as lamina II (Hunt and Mantyh, 2001; Julius and Basbaum, 2001; Snider and McMahon, 1998). Nociceptive neurons innervating visceral targets project to these laminae as well. The convergence of nociceptive afferent fibers, bearing information from diverse peripheral targets, onto a common region of the spinal cord, raises the question of how the brain knows what the body is feeling.

Several viewpoints have emerged to answer this question (reviewed by Craig, 2003). One holds that sensory information about the periphery converges and is then decoded by complex integration occurring at multiple synapses along the ascending pathway to the brain. Another view posits the existence of "labeled lines," segregated synaptic pathways carrying relatively restricted information about stimulus modality to distinct regions of the brain (Han et al., 1998). The tissue origin of somatosensory stimuli may also be segregated by labeled lines (Craig, 2003).

The labeled line hypothesis implies that it should be possible to prospectively identify subsets of sensory neurons carrying information about different stimulus modalities, target tissues, or both. Subpopulations of nociceptors have been identified using physiological (Perl, 1984) or molecular criteria, but retrograde tract tracing and immunohistochemical labeling experiments have thus far failed to link any of these subpopulations to the innervation of a specific end organ (Bennett et al., 1996; Bradbury et al., 1998; Petruska et al., 1997; Wang et al., 1998a, 1998b). Different kinds of sensory endings within individual target tissues can be discriminated using various antigenic markers (Fundin et al., 1997a; Rice et al., 1997), but none of these markers uniquely identifies fibers that innervate a particular target tissue.

Previously, we (Dong et al., 2001) and others (Bender et al., 2002; Lembo et al., 2002) identified a large family of sensory neuron-specific GPCRs, now called *Mas-related G protein-coupled receptors* (*Mrgprs*). In rodent DRG, expression of *Mrgpras*, *Mrgprbs*, *Mrgprc*, and *Mrgprd* defines at least four molecularly distinct neuronal subpopulations (Zylka et al., 2003). All of these neurons are small diameter, suggesting that they are nociceptors. The axis of nociceptor diversity identified by expression of different *Mrgprs* raised the question of whether they might mark distinct nociceptive circuits (Dong et al., 2001), analogous to the olfactory system (Mombaerts et al., 1996). To test this hypothesis, we marked *Mrgprd*-expressing neurons using genetically encoded axonal tracers and examined their central and peripheral projections. Our data reveal that *Mrgprd* is exclusively expressed in nonpeptidergic neurons that innervate the epidermis. Surprisingly, these neurons innervate a specific layer of the epidermis, distinct from that innervated by peptidergic (CGRP$^+$) nerve fibers. These two fiber subtypes project centrally to adjacent laminae of the dorsal spinal cord. Thus, afferent input from different layers of the epidermis is conveyed by topographically segregated sensory circuits. These results are consistent with the idea that at least some aspects of primary sensory information processing may be organized along labeled lines.

*Correspondence: wuwei@caltech.edu

Results

Targeting Axonal Tracers to the *Mrgprd* Locus

Mrgprd is expressed at very high levels by a subset of nonpeptidergic, small-diameter sensory neurons (Dong et al., 2001; Zylka et al., 2003). To map the projections of *Mrgprd*-expressing neurons, we targeted either of two axonal tracers, farnesylated enhanced green fluorescent protein (EGFPf) or human placental alkaline phosphatase (PLAP), to the *Mrgprd* locus by homologous recombination in ES cells (Supplemental Figure S1A at http://www.neuron.org/cgi/content/full/45/1/17/DC1/). PLAP is a well-characterized axonal tracer and is tethered to the extracellular surface of the plasma membrane by a glycophosphatidylinositol (GPI) linkage (Leighton et al., 2001). EGFPf has not previously been used as an axonal tracer in vivo and is bound to the cytoplasmic leaflet of the lipid bilayer by a C-terminal farnesyl group.

We made two mouse lines, in which the entire open reading frame of *Mrgprd* was replaced with an in-frame fusion either of *EGFPf* or of *PLAP* (*Mrgprd*Δ^{EGFPf} and *Mrgprd*Δ^{PLAP}, respectively; Figure 1A; Supplemental Figure S1A at http://www.neuron.org/cgi/content/full/45/1/17/DC1/). We also generated a mouse line in which the *Mrgprd* coding sequence was left intact and followed by an IRES-EGFPf cassette (*Mrgprd*$^{IRES\text{-}EGFPf}$). All targeting events were confirmed by Southern blotting with 5′ and 3′ probes (Supplemental Figure S1B). All lines were fertile and showed no obvious phenotypic or behavioral abnormalities when bred to homozygosity.

To characterize expression of these genetically encoded axonal tracers in *Mrgprd*-expressing neurons, we used antibodies against GFP and PLAP to stain sections of adult DRG. Both markers were expressed in a subset of small-diameter sensory neurons (Figure 1; Supplemental Figures S2A and S2B at http://www.neuron.org/cgi/content/full/45/1/17/DC1/). Furthermore, analysis of *Mrgprd*$\Delta^{EGFPf/PLAP}$ compound heterozygous mice revealed that *Mrgprd* is biallelically expressed (Supplemental Figures S2C and S2F). Therefore, individual neurons in *Mrgprd*$\Delta^{EGFPf/+}$ mice are hemizygous for *Mrgprd* function. In most experiments reported below, we used the gene-deleting EGFPf allele, because expression of EGFPf following the IRES was attenuated ~10-fold relative to the in-frame EGFPf fusion (Supplemental Figures S2A and S2D).

Mrgprd Is Expressed by a Major Subset of Nonpeptidergic Nociceptive Neurons

Unmyelinated nociceptive sensory neurons can be divided into at least two major (albeit partially overlapping) subclasses, based on their expression of neuropeptides and on their growth factor dependence (reviewed in Hunt and Mantyh, 2001; Julius and Basbaum, 2001; Snider and McMahon, 1998). Peptidergic nociceptive neurons contain the neuropeptides CGRP and SUBSTANCE P and require NGF/TRKA signaling for their survival. In contrast, nonpeptidergic neurons lack these peptides, require GDNF/c-RET signaling for their survival, and bind the plant lectin IB4. Peptidergic and nonpeptidergic neurons project to distinct laminae of the dorsal spinal cord (Hunt and Mantyh, 2001; Molliver et al., 1995), but whether their peripheral projections are segregated has not been clear.

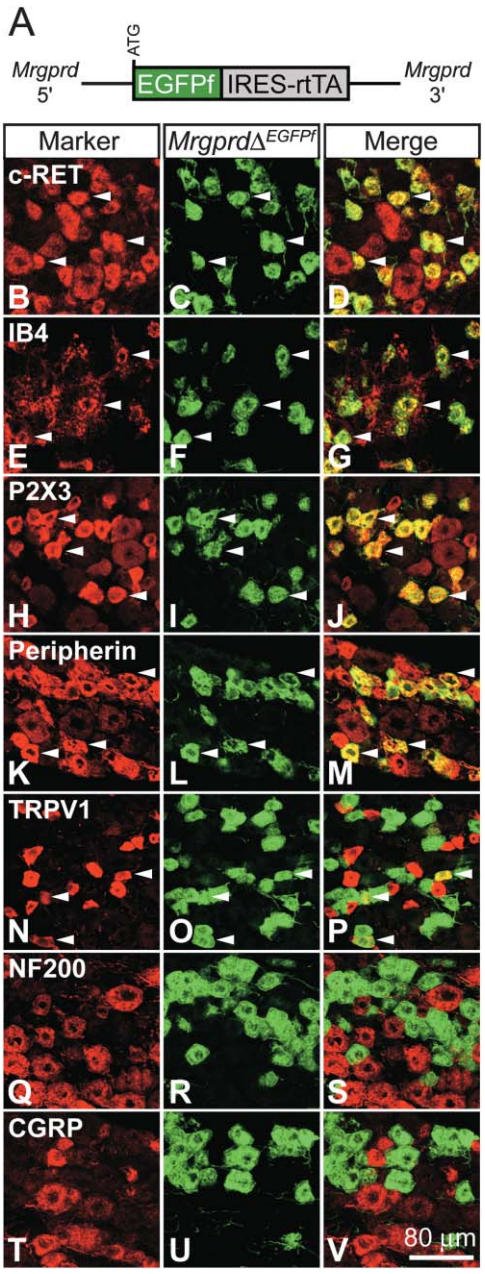

Figure 1. *Mrgprd* Neurons Express Markers Typical of Nonpeptidergic Nociceptive Neurons

(A) *Mrgprd*Δ^{EGFPf} gene-replacing allele.

(B–V) L4-L6 DRG neurons from heterozygous *Mrgprd*Δ^{EGFPf} mice were stained with antibodies against GFP (green) to detect *Mrgprd*-expressing cells and with antibodies against various sensory neuron markers (red). Arrowheads mark examples of double-labeled cells. Scale bar (bottom right) is the same for all panels.

Previous in situ hybridization studies suggested that *Mrgprd*$^{+}$ neurons are contained within the nonpeptidergic subclass (Dong et al., 2001; Zylka et al., 2003). To confirm this assignment, we stained L4-L6 DRG from heterozygous and homozygous *Mrgprd*Δ^{EGFPf} mice with antibodies against GFP and various nociceptive neuron

subtype markers (Figure 1). Virtually all *Mrgprd*$^+$ neurons were labeled with the nonpeptidergic nociceptive markers c-RET and IB4 (Figures 1B–1G). In addition, most if not all *Mrgprd*$^+$ neurons expressed the ATP-gated channel P2X3 and the nociceptive neuron marker PERIPHERIN (Figures 1H–1M).

Importantly, *Mrgprd* neurons represent a subset of nonpeptidergic neurons: 52% of c-RET$^+$ neurons, 76% of IB4 binding neurons, and 59% of P2X3$^+$ neurons express this GPCR. *Mrgprd*$^+$ neurons did not contain neurofilament-200 (NF200) (Figures 1Q–1S), a marker of myelinated axons, consistent with the fact that all *Mrgprd* neurons were unmyelinated when observed by electron microscopy (0.3–1.0 μm axon diameter; Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/45/1/17/DC1/). As expected, the vast majority (>99%) of *Mrgprd*$^+$ neurons expressed low to undetectable levels of CGRP immunoreactivity (Figures 1T–1V). We previously reported (Dong et al., 2001) that *Mrgprd*$^+$ neurons do not express *Trpv1*, the capsaicin and noxious heat-gated receptor (Caterina et al., 1997). However, the more sensitive EGFPf reporter used here revealed colocalization of TRPV1 in a small subset (9%) of *Mrgprd*$^+$ neurons (Figure 1P, arrowheads). The percentage of *Mrgprd*$^+$ neurons expressing these markers was statistically indistinguishable in heterozygous and homozygous *Mrgprd*Δ mice, arguing against a role for *Mrgprd* in maintaining the biochemical phenotype of these neurons. Taken together, these results confirm that *Mrgprd*$^+$ neurons constitute a major subpopulation (75%) of the IB4$^+$ subset of nociceptive sensory neurons.

Mrgprd-Expressing Neurons Exclusively Innervate the Epidermis

Nonpeptidergic IB4$^+$/P2X3$^+$ neurons innervate skin and visceral organs (Bennett et al., 1996; Bradbury et al., 1998; Wang et al., 1998a, 1998b). Since *Mrgprd* was expressed in a subset of IB4$^+$/P2X3$^+$ neurons, we next wanted to determine whether *Mrgprd*$^+$ neurons innervate all known peripheral targets of IB4$^+$/P2X3$^+$ neurons, or rather a subset of these targets. To do this, we examined an extensive array of tissues from heterozygous and homozygous *Mrgprd*Δ newborn and adult animals, both by PLAP histochemistry in *Mrgprd*Δ^{PLAP} mice and by anti-GFP immunohistochemistry in *Mrgprd*Δ^{EGFPf} mice (Supplemental Table S1 at http://www.neuron.org/cgi/content/full/45/1/17/DC1/). As positive controls, we counterstained these tissues for CGRP and PGP 9.5, a panneuronal marker frequently used to identify peripheral nerve fibers (Rice et al., 1997).

Strikingly, in both glabrous and hairy skin, we found that *Mrgprd*$^+$ free nerve endings terminate in the epidermis and do not innervate any specific cutaneous sensory structures (Figures 2A–2D, arrows; Supplemental Table S1 at http://www.neuron.org/cgi/content/full/45/1/17/DC1/). In hairy skin, epidermal *Mrgprd*$^+$ fibers were found near the upper necks of hair follicles, were occasionally seen within bush or cluster endings, and in very rare cases, were observed wrapped around the upper neck of hair follicles as circumferential fibers (Figures 2C and 2D; Supplemental Figure S4). *Mrgprd* expression was not detected in any of the other numerous cutaneous nerve fibers associated with specialized sensory structures, including Meissner corpuscles, Merkel cells, sweat glands, guard hair piloneural complexes, or vibrissa follicle-sinus complexes (Figures 2B–2D; Figure 5B; Supplemental Figure S4). In adult skin, *Mrgprd*$^+$ fibers were also completely absent from muscle and largely absent from blood vessels, tissues that both contain nociceptive afferents (Figures 2E and 2F; Figure 5B; Supplemental Figure S4; Supplemental Table S1).

In the rest of the body, there was a complete absence of *Mrgprd*$^+$ innervation in all other tissues examined, including cornea, meninges, stomach, intestine, muscles, lung, heart, and the bladder (Figures 2A and 2F; Supplemental Table S1 at http://www.neuron.org/cgi/content/full/45/1/17/DC1/). As a positive control, counterstaining was performed with an antibody to CGRP,

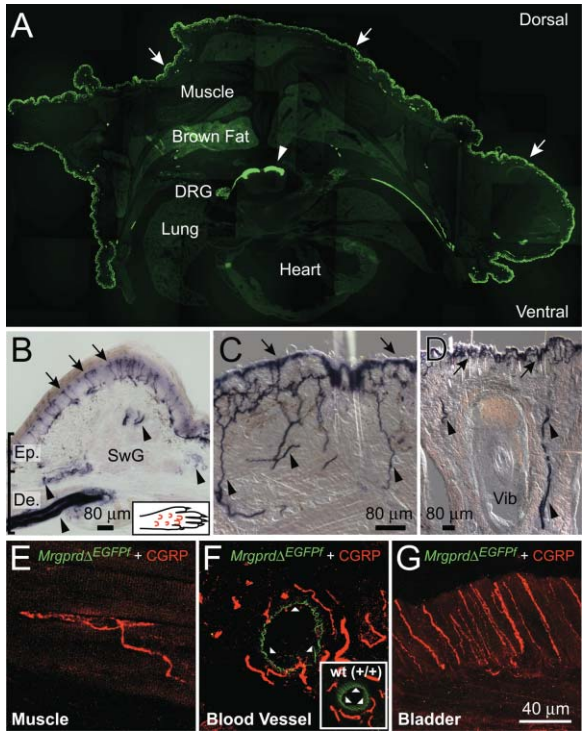

Figure 2. *Mrgprd*-Expressing Neurons Specifically Innervate the Skin

(A) Cross-section through newborn *Mrgprd*Δ^{EGFPf} trunk stained with antibodies to GFP. Arrowhead marks the dorsal horn. *Mrgprd*$^+$ fibers terminate exclusively in the epidermis (arrows). The ventral body skin was removed during perfusion fixation.

(B) Thick glabrous skin of the footpad stained by PLAP histochemistry. (Inset) Mouse hindpaw schematic with the locations of thick glabrous skin indicated in red. Bundles of *Mrgprd*$^+$ fibers can be seen coursing through the dermis (De) and sweat gland (SwG; arrowheads) on their way to the epidermis (Ep). *Mrgprd*$^+$ fibers terminate as free nerve endings (arrows) in the epidermis.

(C and D) Hairy skin of the mystacial pad stained by PLAP histochemistry. *Mrgprd*$^+$ fibers pass through the dermis (arrowheads) and terminate in the epidermis (arrows). The vibrissa follicle-sinus complex (Vib) is unlabeled.

(E–G) Tissues from adult *Mrgprd*Δ^{EGFPf} mice were stained with antibodies to GFP and CGRP. (F) Blood vessel walls were autofluorescent in the green channel in *Mrgprd*Δ^{EGFPf} mice (arrowheads) and in wild-type mice (inset, arrowheads). Scale bar (bottom right) is the same for (E)–(G).

which labeled peptidergic nociceptive fibers known to innervate these structures (Figures 2E–2G). Notably, since nonpeptidergic nociceptive neurons also innervate many of these tissues, such as the bladder (Bennett et al., 1996; Cockayne et al., 2000; Wang et al., 1998b), these results imply the existence of additional, *Mrgprd*-negative, nonpeptidergic nociceptive afferents. *Mrgprd*$^+$ neurons from heterozygous and homozygous *Mrgprd*Δ mice exhibited the same highly restricted pattern of epidermal innervation, indicating that *Mrgprd* gene function is not necessary for proper axon guidance to the periphery.

Mrgprd$^+$ and CGRP$^+$ Fibers Exhibit Distinct Morphologies and Termination Zones within the Epidermis

The epidermis is complex, consisting of four distinct keratinocyte-derived layers, and receives both peptidergic and nonpeptidergic nociceptive innervation (Rice et al., 1997). It has been difficult to directly compare the pattern of epidermal innervation by these two fiber subtypes, because identification of nonpeptidergic nerve endings in skin is obscured by IB4 staining of keratinocytes (Rice, 1993). Furthermore, histochemical staining for fluoride-resistant acid phosphatase (FRAP), the only other definitive marker for nonpeptidergic neurons, does not reveal the morphology of individual fibers in the periphery (Hunt and Rossi, 1985; Silverman and Kruger, 1988).

To determine whether *Mrgprd*$^+$ fibers and peptidergic fibers innervated common or distinct regions of the epidermis, we compared their distribution using antibodies to GFP and CGRP, respectively. Double labeling with these markers revealed two largely distinct nerve fiber subpopulations, which exhibited different morphologies and termination zones. *Mrgprd*$^+$ fibers coursed fairly directly through the stratum basalis and stratum spinosum, then meandered extensively amongst the keratinocytes of the stratum granulosum, and ultimately terminated $\sim$10 μm from the stratum corneum (Figures 3A, 3C, and 3F; Figure 5, green). In contrast, CGRP$^+$ fibers generally exhibited a relatively straight trajectory and terminated in the underlying stratum spinosum (Figures 3B and 3C; Figure 5, red).

We occasionally observed fibers that appeared to be positive for both *Mrgprd*-EGFPf and CGRP expression (Figure 3C, arrowheads); however, closer examination revealed that these were actually distinct but closely intertwined fibers of each subtype (Figure 3D). Quantification indicated that, on average, $\sim$10% of all *Mrgprd*-expressing fibers were intertwined with CGRP$^+$ fibers and, conversely, that $\sim$20% of all CGRP$^+$ fibers were intertwined with *Mrgprd*-expressing fibers, although these percentages were different in thin versus thick (dermal papilla-containing) glabrous skin (cf. Figure 3C versus Figure 3E). Interestingly, in such cases of intertwining, CGRP$^+$ fibers appeared to penetrate into the stratum granulosum, the termination zone occupied primarily by *Mrgprd*$^+$ fibers (Figure 3C). Efforts to determine whether the CGRP$^+$ fibers that intertwined with *Mrgprd*$^+$ fibers could be distinguished from solitary CGRP$^+$ fibers, by the expression of other markers, were inconclusive. These data thus reveal a coinnervation of identical cutaneous domains by molecularly distinct but intimately intertwined unmyelinated nerve fibers.

We quantified the proportion of total free nerve endings represented by *Mrgprd*$^+$ and CGRP$^+$ fibers in the epidermis. Approximately 60% of the fibers in glabrous skin were *Mrgprd*$^+$, whereas $\sim$40% were CGRP$^+$, with no quantitative differences between heterozygous and homozygous *Mrgprd*Δ animals. Staining for EGFPf and the panaxonal marker PGP 9.5 revealed that, similarly, $\sim$60% of all PGP 9.5$^+$ epidermal free nerve endings were EGFPf$^+$, whereas $\sim$40% were EGFPf$^-$/PGP 9.5$^+$ (Figures 3F–3H). These data suggested that virtually all of the free nerve endings in the epidermis can be accounted for by either *Mrgprd*$^+$ or CGRP$^+$ fibers.

To determine if there were any additional epidermal fibers not marked by *Mrgprd* or CGRP, we next stained hairy and glabrous skin with a cocktail of antibodies against GFP (*Mrgprd*-fibers), CGRP, and NF200 (to mark myelinated fibers) and counterstained with PGP 9.5. Remarkably, 98% (827/841) of the PGP 9.5$^+$ epidermal fibers in glabrous skin were *Mrgprd*$^+$/CGRP$^+$/NF200$^+$, whereas only 2% of the epidermal fibers were PGP 9.5$^+$ and not stained with the antibody cocktail (gray fibers in Figure 5B). In contrast, many PGP 9.5$^+$ but EGFPf/CGRP/NF200-negative fibers were observed in muscle and sweat glands (Figure 5B), as expected. These results indicate that 98% of all epidermal fibers are either *Mrgprd*$^+$ or CGRP$^+$ (NF200 does not significantly label epidermal fibers). Taken together, these results demonstrate that *Mrgprd*-expressing neurons represent the predominant subset of epidermal free nerve endings (60%) and likely constitute all nonpeptidergic innervation of the epidermis.

Mrgprd$^+$ and CGRP$^+$ Neurons Project to Distinct Laminae in the Dorsal Spinal Cord

The observation that *Mrgprd*$^+$ and CGRP$^+$ neurons project to distinct layers of the epidermis raised the question of whether they project to different laminae in the dorsal spinal cord. Peptidergic sensory neurons are known to project predominantly to lamina I and outer lamina II (II$_o$) (McNeill et al., 1988; Snider and McMahon, 1998), while nonpeptidergic (IB4$^+$) neurons have been proposed to terminate in inner lamina II (II$_i$) (Hunt and Mantyh, 2001; Silverman and Kruger, 1990; Snider and McMahon, 1998). However, recent studies have suggested that IB4$^+$ neurons may instead project to lamina II$_o$ (Woodbury et al., 2000), implying that some peptidergic and nonpeptidergic neurons may coterminate. We examined the laminar termination of *Mrgprd*$^+$ neurons by staining spinal cord sections from heterozygous or homozygous *Mrgprd*Δ^{EGFPf} animals with an antibody to GFP, together with fluorescent lectin IB4, or an antibody to CGRP. *Mrgprd*$^+$ fibers projected to a narrow lamina in the dorsal horn that overlapped the IB4$^+$ lamina and was below the CGRP$^+$ lamina (Figures 4A–4C). Some intermingling of *Mrgprd*$^+$ and CGRP$^+$ fibers was observed, but almost no overlap at the single-fiber level was detected by confocal microscopy (Figure 4C, boxed region). This is consistent with lack of overlap of *Mrgprd* and CGRP expression in cell bodies within the DRG.

Surprisingly, the vast majority of *Mrgprd*$^+$ fibers terminated dorsal to the region expressing PKCγ, a marker of lamina II$_i$ and III interneurons (Figure 4D) (Malmberg et al., 1997; Mori et al., 1990; Polgar et al., 1999). Thus,

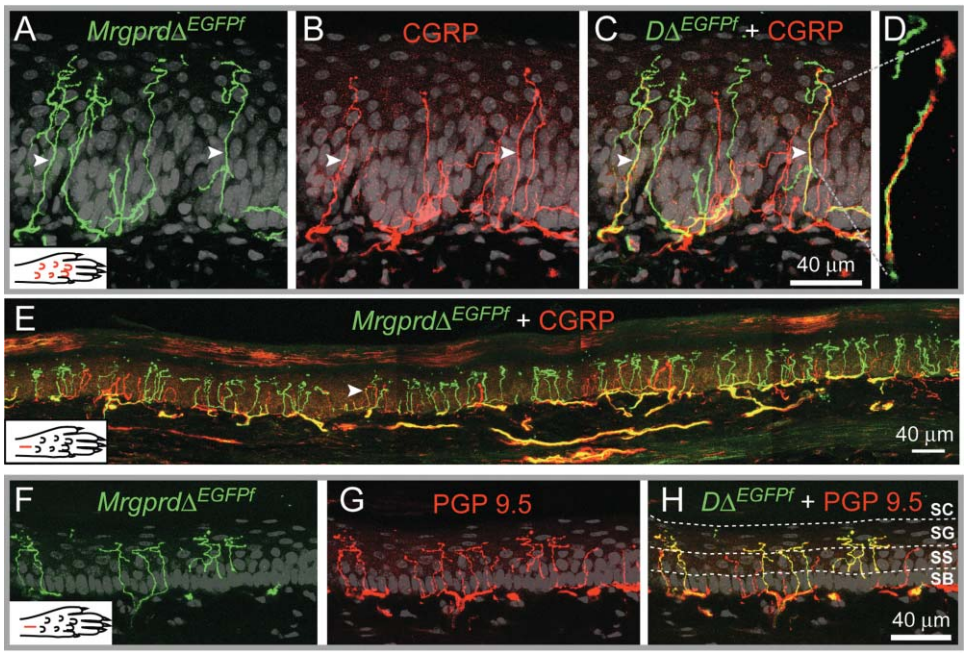

Figure 3. *Mrgprd* Fibers Represent a Morphologically Distinct Class of Epidermal Endings that Are Intertwined with Peptidergic Fibers in Glabrous Skin

(A–C) Confocal image of (A) *Mrgprd* and (B) peptidergic, CGRP-containing, free nerve endings in thick epidermis of glabrous skin from *MrgprdΔ*^{EGFPf} mice. (Inset in [A]) Mouse hindpaw schematic with the locations of thick glabrous skin indicated in red. Arrowheads mark intertwined fibers. Nuclei are pseudocolored gray to highlight cellular stratification within the epidermis.

(D) Enlarged image, ~0.5 μm single confocal optical section through an intertwined fiber pair. Note the clear separation between red and green colors.

(E) Photomontage spanning thin epidermis of glabrous skin. (Inset) Approximate anatomical location of this image is indicated in red. The density of intertwined *Mrgprd* and CGRP fibers (arrowhead) is lower in thin glabrous skin. *Mrgprd* fibers display a more torturous, zigzag shape compared with CGRP-containing fibers.

(F–H) All *Mrgprd*$^+$ epidermal fibers terminate in the stratum granulosum (SG) and are labeled by the panneuronal fiber marker PGP 9.5. There are also several PGP$^+$, *Mrgprd*-negative epidermal fibers (red fibers in [H]) that terminate in the stratum spinosum (SS), likely peptidergic. SB, stratum basalis; SC, stratum corneum. The epidermal subdivisions were identified by keratinocyte nuclear morphology and packing density.

Mrgprd$^+$ fibers were interpolated between the laminae defined by CGRP and PKCγ labeling (Figure 4E) and were coextensive with IB4$^+$ fibers (Figure 4B). Since CGRP$^+$ fibers are considered to project to both lamina I and lamina II$_o$ (McNeill et al., 1988), while PKCγ marks lamina II$_i$, these data suggest that *Mrgprd*$^+$/IB4$^+$ fibers project to neither lamina II$_o$ nor II$_i$, as classically defined. Whether this projection field should be considered a subdivision of lamina II$_o$ (Woodbury et al., 2000) or of lamina II$_i$ (Hunt and Mantyh, 2001; Silverman and Kruger, 1990; Snider and McMahon, 1998) or should be assigned a new name (e.g., lamina II$_{middle}$) remains to be decided. The projection of *Mrgprd*$^+$ fibers was similar in spinal cords from heterozygous and homozygous *Mrgprd*

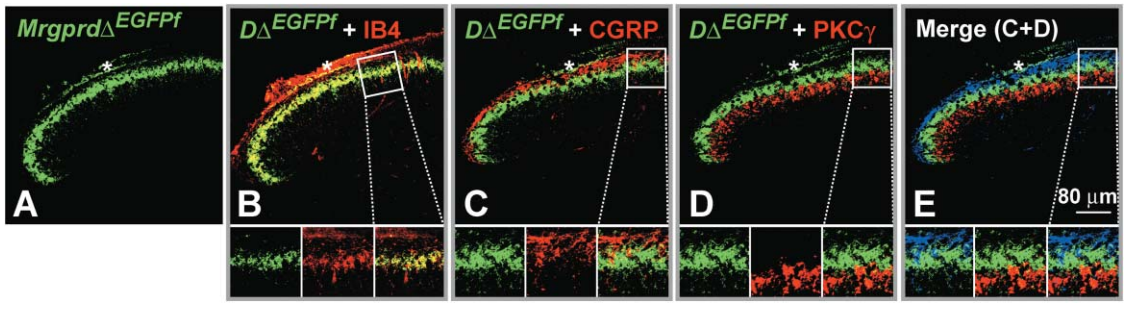

Figure 4. *Mrgprd* Axons Terminate in the Nonpeptidergic Lamina of the Spinal Cord Dorsal Horn

Confocal images of the L4-L6 region of adult spinal cord from homozygous *MrgprdΔ*^{EGFPf} mice. (A and B) *Mrgprd*$^+$ axons and IB4 binding axons terminate in the same lamina of the spinal cord. (C) *Mrgprd*$^+$ and CGRP$^+$ fibers do not substantially overlap in the dorsal horn but do intermingle. (D) *Mrgprd*$^+$ axons do not substantially overlap with PKCγ$^+$ interneurons. (E) Triple-labeled image shows the relationship between *Mrgprd*$^+$ and CGRP fibers (pseudocolored blue, from [C]) and PKCγ interneurons. The boxed regions in (B)–(E) were magnified and arranged at the bottom as individual and merged color channels. Asterisks mark Lissauer's tract.

knockout animals, indicating that the *Mrgprd* gene is not essential for guidance of *Mrgprd*$^+$ axons into the dorsal horn.

Discussion

Our data identify two populations of nociceptive sensory neurons with topographically distinct projections to the epidermis and demonstrate that they project centrally to different laminae of the dorsal spinal cord. Expression of *Mrgprd* identifies a subset of IB4$^+$ neurons that exclusively project to the skin, among all possible tissues receiving nociceptive innervation. Moreover, within the skin, *Mrgprd*$^+$ fibers selectively innervate the epidermis and are absent from the dense network of nerves innervating specialized sensory structures such as hair follicles, Merkel cells, Meissner's corpuscles, and mystacial vibrissae. This extraordinary level of target specificity is completely unexpected, based on earlier neuroanatomical tracing studies. *Mrgprd*$^+$ fibers constitute 60% of all epidermal free nerve endings and terminate in a highly restricted zone, the stratum granulosum. Surprisingly, CGRP$^+$ fibers, which constitute the only other major population of epidermal free nerve endings, terminate in the underlying stratum spinosum. These data, therefore, reveal a topographic segregation of the projections of peptidergic and nonpeptidergic nociceptive sensory neurons to the epidermis. The fact that these fibers project centrally to different laminae of the dorsal spinal cord (Hunt and Mantyh, 2001; Hunt and Rossi, 1985) indicates, moreover, that this peripheral topographic segregation is preserved at the first relay station in the CNS.

Mrgprd Reveals a Dedicated Afferent Pathway from the Epidermis

It has been widely assumed that DRG contain a subpopulation of nociceptive neurons with exclusively cutaneous peripheral targets. Nevertheless, despite a wealth of molecular markers that distinguish different subsets of small-diameter sensory neurons (Julius and Basbaum, 2001), efforts to find genes that uniquely identify such cutaneous afferents—or indeed any population with a restricted peripheral target specificity—have been unsuccessful. Retrograde tracing studies have suggested that, as a population, IB4$^+$ neurons innervate both skin and visceral organs (Bennett et al., 1996; Bradbury et al., 1998; Petruska et al., 1997; Wang et al., 1998a, 1998b). Whether these cutaneous and visceral afferents represent distinct subpopulations of IB4$^+$ neurons has not been clear. The marking of *Mrgprd*-expressing neurons with genetically encoded axonal tracers provides unambiguous evidence for a subpopulation of IB4$^+$ neurons that project exclusively to the epidermis. More generally, our results identify a subclass of nociceptive neurons that innervate a single target tissue.

In the rat and mouse, there are at least four distinct subsets of small-diameter sensory neurons defined by expression of different *Mrgpr* family members, and most of these fall within the nonpeptidergic subpopulation (Dong et al., 2001; Zylka et al., 2003). Since *Mrgprd* marks the vast majority of nonpeptidergic projections to the epidermis, and since the nonpeptidergic population

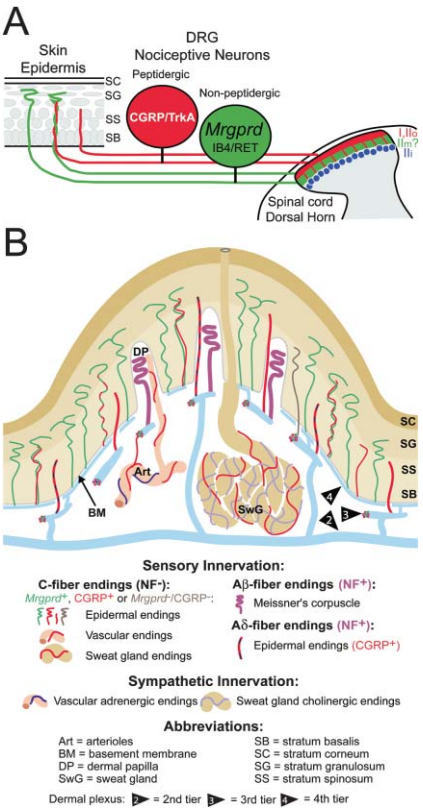

Figure 5. Summary of Sensory Circuitry

(A) Nociceptive information is carried by molecularly distinct and parallel neuronal circuits (red and green). These parallel circuits terminate centrally within adjacent lamina of the spinal cord and terminate peripherally in different zones of the epidermis as free nerve endings with distinct morphologies. Peptidergic and nonpeptidergic fibers are occasionally intertwined, suggesting intercommunication peripherally. CGRP$^+$ fibers that do enter the SG are frequently intertwined with *Mrgprd*$^+$ fibers. The location of PKCγ^+ interneurons (blue) in lamina II$_i$ is also shown.

(B) Innervation in glabrous skin. Includes our current findings in relationship to previous studies of innervation within mouse skin (Fundin et al., 1997b; Rice et al., 1998). The SG is differentially shaded. In rare cases, we could not conclusively determine if *Mrgprd* and CGRP fibers were intertwined or if these markers were in the same fiber (red and green striped fibers). The schematic does not reflect the relative proportions of each fiber type, nor does it illustrate Aβ-fiber innervation, which would terminate on Merkel cells in SB of the epidermis.

also contains neurons projecting to other tissues, those *Mrgprs* not coexpressed with *Mrgprd* may well mark sensory neuronal subsets that project to noncutaneous tissues, such as visceral organs. Targeting axonal tracers to these other *Mrgpr* genes should reveal the nature of these projection targets, the degree of end organ specificity exhibited by different *Mrgpr*-expressing subpopulations, and whether these subpopulations have distinct projection targets in the spinal cord.

Mrgprd Expression Reveals Topographically Segregated Patterns of Peptidergic and Nonpeptidergic Innervation of the Epidermis

It has long been appreciated that peptidergic and nonpeptidergic neurons comprise two largely distinct, par-

allel pain circuits (Hunt and Mantyh, 2001; Hunt and Rossi, 1985). These neurons are known to project to distinct laminae in the dorsal spinal cord (Molliver et al., 1995; Silverman and Kruger, 1990), but whether these circuits convey distinct sensory information from the periphery has not been clear. Our studies reveal a topographically segregated pattern of cutaneous innervation by these two populations of fibers. The nonpeptidergic free nerve endings that are labeled by *Mrgprd-EGFPf* terminate in the stratum granulosum of the epidermis, while most CGRP$^+$ fibers terminate in the subadjacent stratum spinosum (Figure 5). This pattern is consistent with emerging evidence that the epidermis has a concomitantly stratified functional chemistry (Khodorova et al., 2003). The topographic segregation of the peripheral projections of peptidergic and nonpeptidergic neurons had not been realized previously, because the two best characterized nonpeptidergic markers, FRAP and IB4, are for technical reasons not useful for identifying individual fibers in the epidermis (Hunt and Rossi, 1985; Rice, 1993; Silverman and Kruger, 1988). The fact that we observe the same pattern of peripheral innervation using two distinct axonal tracers, PLAP and EGFPf, makes it highly unlikely that this pattern is an artifact of the tracer used. Thus, this genetic marking approach has revealed that the well-known laminar segregation of the central projections of peptidergic and nonpeptidergic neurons reflects a corresponding segregation of their peripheral projections as well (Figure 5A).

The elucidation of this segregated pattern of cutaneous innervation begs the question of the nature of the information carried by these two parallel nociceptive circuits. Peptidergic and nonpeptidergic neurons have generally been thought to differ in the adaptive pain responses that they mediate (inflammatory versus neuropathic) (Julius and Basbaum, 2001). Our data suggest that there may be additional functional distinctions between these subpopulations that have previously escaped detection. The ability to genetically ablate or silence these distinct pathways (Gogos et al., 2000; Yu et al., 2004) should reveal whether they have different sensory functions in vivo.

While most *Mrgprd*$^+$ and CGRP$^+$ nerve endings have distinct morphologies and termination zones in the skin, in ~10%–20% of cases we observed close intertwining of these fibers. This observation is consistent with prior reports of close associations between unmyelinated peptidergic and nonpeptidergic axons (Guo et al., 1999, 2001; Hunt and Rossi, 1985; Ulrich-Lai et al., 2001; Wang et al., 1998a) and raises the question of whether there are any functional interactions between these fibers. There is some indirect evidence suggesting that intertwined fibers may communicate with one another in the skin and in visceral organs (Guo et al., 1999, 2001; Pare et al., 2001; Ulrich-Lai et al., 2001). In addition, nociceptive neurons also release neuropeptides, like CGRP and SUBSTANCE P, from their peripheral terminals into the skin and visceral organs. These peripherally released neuropeptides contribute to neurogenic inflammation and sensitization (Hunt and Mantyh, 2001; Julius and Basbaum, 2001). Intertwined peptidergic and nonpeptidergic nociceptive fibers may thus have the capacity to cross-activate, sensitize, or desensitize one another under different conditions. If so, then the well-accepted polymodal sensitivity of cutaneous nociceptors could, in some cases, reflect intercommunication between two intertwined fibers, each of which is more specifically tuned.

How Does the Brain Know What the Body Is Feeling?

Sensory input from different regions of the body is topographically mapped to different regions of somatosensory and insular cortex, but how the stimulus modality and tissue origin of this information are decoded during transfer from the periphery has been controversial. The "labeled line" hypothesis proposes the existence of anatomically and molecularly distinct circuits that convey different stimulus modalities, such as noxious heat verses cooling (Han et al., 1998), and perhaps different tissue origins as well (Craig, 2003). Since expression of *Mrgprd* marks a subpopulation of sensory neurons that exclusively innervate the epidermis, activation of these neurons should tell the brain that the epidermis, and no other sensory organ, has been stimulated. The existence of such a dedicated projection raises the question of whether the postsynaptic targets of these neurons in the spinal cord are similarly dedicated to exclusively processing epidermal afferent input. Tracing of the second- and third-order projections of *Mrgprd*$^+$ neurons (Horowitz et al., 1999; Yoshihara et al., 1999), and comparison of these circuits to those engaged by other subpopulations of sensory neurons with distinct peripheral target specificities, should shed light on the degree to which sensory processing pathways from different end organs remain segregated from the body to the brain.

Experimental Procedures

The generation of gene-targeted mice and immunohistochemical staining were performed using established procedures. Further experimental details can be found on the web (http://www.neuron.org/cgi/content/full/45/1/17/DC1/).

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